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A NUCLEAR MAGNETIC RESONANCE STUDY OF METABOLISM IN THE FERRET HEART DURING HYPOXIA AND INHIBITION OF GLYCOLYSIS

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SUMMARY

1. ^{31}P nuclear magnetic resonance was used to measure the relative concentrations of phosphorus-containing metabolites in Langendorff-perfused ferret hearts. Intracellular concentrations of inorganic phosphate ($[Pi]_i$), phosphocreatine ($[PCr]_i$), ATP ($[ATP]_i$) and H^+ (pH_i) were monitored under control conditions and while oxidative phosphorylation and/or glycolysis were prevented. Mechanical performance was assessed by recording the pressure developed in a balloon placed in the left ventricle.

2. Oxidative phosphorylation was prevented either by replacement of O_2 with N_2 or by addition of cyanide. When the rate of oxidative phosphorylation was reduced by either method, developed pressure fell to a stable level of about 35% of control after 5 min. The pH_i (control value 6·98) first increased to a peak of 7·07 after 2 min but then decreased to give a stable acidosis (pH 6·85). $[PCr]_i$ decreased rapidly to about 15% of the control value after 5 min whereas $[ATP]_i$ declined very slowly, reaching about 90% of the control value after 10 min.

3. Reduction in the rate of glycolysis was achieved either (i) by removal of external glucose and depletion of glycogen stores by a long (1–2 h) period of stimulation or (ii) by removal of glucose and application of 2-deoxyglucose (1 mM) for 30–60 min. These procedures had only a small effect on pressure development, $[ATP]_i$, $[PCr]_i$ and pH_i. Measurements of lactate production showed that these procedures reduced the rate of glycolysis by a factor of about 10.

4. When oxidative phosphorylation was prevented during periods when the rate of glycolysis was reduced, developed pressure fell to less than 5% of control after 5 min and there was a subsequent increase in resting pressure (hypoxic contracture). pH_i (control value 7·03) first increased to a peak of 7·12 and then declined to about pH 7·00, but there was no subsequent acidosis. $[PCr]_i$ fell rapidly to about 10% of control after about 5 min while $[ATP]_i$ declined to about half of its control value over 10 min.

5. It is concluded that (i) when oxidative phosphorylation alone is prevented, the

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changes in pH_i can account for a substantial part of the changes in developed pressure. The increase in $[Pi]_i$ probably also contributes to the decline of developed pressure. (ii) When oxidative phosphorylation was prevented under conditions in which the rate of glycolysis was also reduced, the more pronounced decline in developed pressure which occurs within 5 min cannot be accounted for by pH_i changes and is probably not explained by the rise in $[Pi]_i$ or by the moderate fall of $[ATP]_i$. The possibility that a fall in free energy change of hydrolysis of ATP may account for the decline of developed pressure under these conditions is considered but the evidence is equivocal. The hypoxic contracture which is subsequently observed under these conditions is probably adequately accounted for by the fall in $[ATP]_i$, which reaches low levels at about the same time.

INTRODUCTION

Hypoxia or ischaemia lead to a rapid and pronounced decline in tension development by heart muscle. The mechanisms which underlie this early hypoxic contractile failure are still not established (for review see Gibbs, 1978; Nayler, Poole-Wilson & Williams, 1979). However, recent measurements of intracellular calcium (Allen & Orchard, 1983*b*) have suggested that different mechanisms are involved, depending on whether or not glycolysis can occur during the hypoxic period.

In the well-oxygenated heart the pathways for ATP production are almost entirely aerobic. The substrate for oxidative metabolism can be (i) exogenous glucose or endogenous glycogen (aerobic glycolysis), (ii) intermediary metabolites such as pyruvate, lactate or acetate, and (iii) exogenous fatty acids or endogenous lipids (Neely & Morgan, 1974; Randle & Tubbs, 1979). When oxidative phosphorylation is blocked, provided glucose or glycogen is available, there is a large increase in anaerobic glycolysis which produces ATP and lactate (Williamson, 1966): the latter leads to intracellular acidosis. Allen & Orchard (1983*b*) measured the rise in intracellular calcium concentration associated with contraction (the calcium transient) under these conditions and found that the amplitude of the calcium transients did not change. This result was consistent with the hypothesis (Katz & Hecht, 1969) that the rapid decline of developed tension in ischaemia is due to an effect of acidosis on the sensitivity of the contractile proteins to calcium (Fabiatio & Fabiato, 1978; Allen & Orchard, 1983*a*) and not due to changes in calcium entry or release associated with activation (Nayler *et al.* 1979). However, quantitative assessment of this hypothesis requires simultaneous measurements of intracellular pH (pH_i).

Prevention of oxidative phosphorylation when the rate of glycolysis has been reduced produces a rapid and complete decline in developed tension. This decrease in developed tension is associated with, and probably a consequence of, a similar decrease in the calcium transients (Allen & Orchard 1983*b*). Current evidence suggests that neither the change in pH_i nor the small decline in intracellular ATP concentration ($[ATP]_i$) (Williamson, 1966; Hearse, 1979; Matthews, Radda & Taylor, 1981) can account for this decline in the calcium transients. For this reason several groups (Kammermeier, Schmidt & Jungling, 1982; Allen & Orchard, 1983*b*) have suggested that under these conditions there may be decrease in the free energy change of hydrolysis of ATP (ΔG_{ATP}). The magnitude of ΔG_{ATP} places an upper limit on the

concentration gradient of calcium which the sarcoplasmic reticulum pump can achieve between the myoplasm and the sarcoplasmic reticulum. Consequently, if ΔG_{ATP} falls sufficiently, less calcium will be pumped into the sarcoplasmic reticulum and less will be available for release and for the initiation of contraction.

In the present studies we have therefore monitored pH_i, intracellular phosphocreatine concentration ($[PCr]_i$), and $[ATP]_i$ using ^{31}P nuclear magnetic resonance while (i) inhibiting oxidative phosphorylation, (ii) inhibiting glycolysis and (iii) inhibiting both glycolysis *and* oxidative phosphorylation. Although the ΔG_{ATP} cannot be measured directly in these studies, its value can be calculated from our results with certain assumptions (Dawson, Gadian & Wilkie, 1978).

Preliminary accounts of parts of this work have appeared elsewhere (Allen, Morris & Orchard, 1983; Orchard, Allen & Morris, 1984).

METHODS

Small ferrets were anaesthetized with pentobarbitone. The heart was removed rapidly, washed in Tyrode solution (see below) and weighed. Langendorff perfusion with Tyrode solution at 30 °C was started within 1–2 min. Perfusion was at constant flow, which has the advantage that the myocardial supply of O_2 is constant and under experimental control. Perfusion pressure was monitored with a transducer connected to the inflow line. A glass filter (porosity no. 3) was placed in the inflow line to prevent particulate matter entering the capillary network. Further details of the perfusion apparatus are given in Morris, Allen & Orchard (1984).

The sino-atrial node was located and either crushed or excised. Stimulating electrodes were pushed into the left ventricular wall, and pacing of the heart commenced at a rate which suppressed spontaneous contractions, generally 1–2 Hz. The stimuli were 5 ms pulses at 1·5 × threshold. A latex balloon was passed into the left ventricle and the pressure in this balloon was monitored using a second pressure transducer. The balloon, tubing and transducer were all filled with fluid so that the contractions of the left ventricle were isovolumic. Perfusion pressure and left ventricular balloon pressure were displayed on a two-channel pen recorder.

The preparation was lowered into a spectrometer sample tube (diameter 25 mm). The hearts used were sufficiently small (4–5 g) that they did not press on the sides of this tube. Effluent perfusate was removed from the tube at a level higher than the top of the heart so that the preparation was fully immersed. After discarding the initial 300–400 ml, the perfusate was recycled.

The hearts were perfused at a flow rate of 4–8 ml (g wet wt.)$^{-1}$ min^{-1} which produced a mean perfusion pressure of 50–100 mmHg. This flow rate was felt to be adequate on the following grounds: (i) it provides 0·07–0·14 ml O_2 (g wet wt.)$^{-1}$ min^{-1} and at the low stimulation rate and temperature used in this study the O_2 consumption is probably about 0·05 ml (g wet wt.)$^{-1}$ min^{-1} (see discussion in Gibbs, 1978); (ii) in a few experiments the P_{O_2} of perfusate leaving the heart was measured and found to be about 200 mmHg (P_{O_2} of perfusate entering the heart was 550–600 mmHg); (iii) in two experiments lactate production was measured (using a linked NAD/lactate dehydrogenase assay, Sigma Chemical Co.) and found to average 1·1 μmol (g wet wt.)$^{-1}$ min^{-1} at a flow of 1 ml (g wet wt.)$^{-1}$ min^{-1}, 0·5 μmol (g wet wt.)$^{-1}$ min^{-1} at 2 ml (g wet wt.)$^{-1}$ min^{-1}, 0·15 μmol (g wet wt.)$^{-1}$ min^{-1} at 4 ml (g wet wt.)$^{-1}$ min^{-1} and 0·12 μmol (g wet wt.)$^{-1}$ min^{-1} at 8 ml (g wet wt.)$^{-1}$ min^{-1}. Thus, anaerobic glycolysis is minimal at flow rates of 4–8 ml (g wet wt.)$^{-1}$ min^{-1}.

Mechanical performance of the heart was assessed by measuring developed (peak minus resting) pressure and resting pressure. In order that the mechanical data should be directly comparable with the metabolic data, (i) the pressure measurements and the nuclear magnetic resonance (n.m.r.) data were averaged over the same 1 min periods, and (ii) all pressure measurements in any one experiment were normalized to the control value obtained when the control n.m.r. data were acquired.

Under the conditions of our experiments the hearts showed a slow mechanical and metabolic decline. Typically, over a 6 h experiment developed pressure would have declined by up to 50% and there would be some loss of total phosphate signal from the heart. Some of this decline may

be associated with the interstitial oedema which developed because of the absence of protein in our perfusate. The interventions shown in Figs. 2–7 lasted only 15 min and over this period the decline was negligible.

Solutions

The solution with which the heart was initially perfused (standard Tyrode) contained (mM): Na^+, 135; K^+, 5; Mg^{2+}, 1; Ca^{2+}, 2; Cl^-, 104; HCO_3^-, 20; HPO_4^{2-}, 1; acetate, 20; glucose, 10; insulin, 4×10^{-5}, and was equilibrated with 95 % O_2/5 % CO_2 to give a final pH of 7·4. In order to determine pH_i it is necessary to be able to identify intracellular inorganic phosphate (Pi), so that this could be done unequivocally, Pi was removed from the perfusate when measurements of pH_i were undertaken (Salhany, Pieper, Wu, Todd, Clayton & Eliot, 1979). Periods of external Pi removal were generally shorter than 1 h. In control experiments removal of external Pi had no observable effect on mechanical performance, $[ATP]_i$, $[PCr]_i$ or internal Pi concentration ($[Pi]_i$) for periods of up to 1 h (cf. Morris *et al.* 1984).

Cyanide solutions were freshly made up from NaCN and buffered to pH 7·4 with HEPES shortly before use (see Allen & Orchard, 1983*b*, for details).

N.m.r. measurement of metabolites

A general account of biological applications of n.m.r. is given by Gadian (1982) and application of the techniques to the perfused heart have been described by Gadian, Radda, Richards & Seeley (1979) and Morris *et al.* (1984). We used a Bruker WM 200 spectrometer with a field strength of 4·7 T. Brief, radiofrequency (20 μs, 81 MHz) pulses were generated across the heart at 1 s intervals. The spectrum resulting from one pulse is too noisy to be useful, and in most of our experiments the result of sixty pulses at 1 s intervals have been averaged. Thus, the minimum time resolution is 1 min.

Although the necessity of averaging makes it desirable to obtain scans at frequent intervals, this results in a variable decrease in the size of peaks in the spectra. This is because the time taken for a phosphorus nucleus to relax to its resting state after a pulse is several seconds and depends on the chemical environment of the individual phosphorus nucleus. The magnitude of this decrease (the saturation factor) was determined by comparing the area under a given peak when the time between pulses was long enough for complete recovery (8 s) with the area under the same peak using pulses at 1 s intervals.

Saturation factors were determined in eleven experiments and the mean $\pm$ S.E. of the mean were as follows: sugar phosphates, $1{\cdot}43 \pm 0{\cdot}19$; Pi, $1{\cdot}34 \pm 0{\cdot}10$; PCr, $1{\cdot}70 \pm 0{\cdot}07$; α-ATP, $1{\cdot}11 \pm 0{\cdot}07$; β-ATP, $1{\cdot}17 \pm 0{\cdot}04$; γ-ATP, $1{\cdot}08 \pm 0{\cdot}06$. These saturation factors were used to correct the areas obtained with 1 s between pulses when plotting the graphs of relative concentrations.

Calibration of metabolite concentrations

The identification of the major peaks in the ^{31}P n.m.r. spectrum from the heart is now well established (Gadian, 1982; Dawson & Wilkie, 1984) and they are shown in Fig. 1. Other compounds of interest are known from solution chemistry to have the following chemical shifts in parts per million (p.p.m.) relative to PCr at pH 7·0 (Gadian *et al.* 1979): sugar phosphates, +6 to +7; AMP, +6·3; inosine monophosphate (IMP), +6·3; α-ADP, −7·0; β-ADP, −3·0; NADH, −8·1; NAD^+, −8·3. Notice that the β-ATP peak is the only ATP peak which is not contaminated by other peaks; consequently we have used the area of the β-ATP peak to estimate $[ATP]_i$. The peak labelled γ-ATP in Fig. 1 has contributions from both ATP and ADP; consequently we have measured γ-ATP − β-ATP to estimate $[ADP]_i$.

After correction for saturation factors, the area under any peak is directly related to the amount of this compound which is mobile and which lies within the sensitive volume of the spectrometer. Areas were measured on spectra using a computerized planimetry system after estimation of the base line on either side of the peak by eye. However, it is still not straightforward to convert these areas into absolute concentrations because of (i) uncertainties about the region and volume of distribution of the various compounds, (ii) uncertainties about the size and uniformity of the sensitive region in the spectrometer (for fuller discussion see Dawson & Wilkie, 1984). For these reasons we present the results as relative concentration of the various metabolites. In each experiment the area under the β-ATP peak (corrected for saturation factor) at the start of the collection of spectra (typically 30–60 min after removal of the heart) has been arbitrarily defined as unit

concentration (see below for absolute value). All concentrations for each substance throughout each experiment have been normalized to this value. ATP was chosen for this purpose because its concentration is held nearly constant under control conditions by the buffering action of PCr and creatine kinase (cf. Carlson & Wilkie, 1974).

We also measured the absolute magnitude of [ATP] in ferret hearts under identical conditions to the above. Three ferret hearts were freeze-clamped and the ATP measured by high-pressure liquid

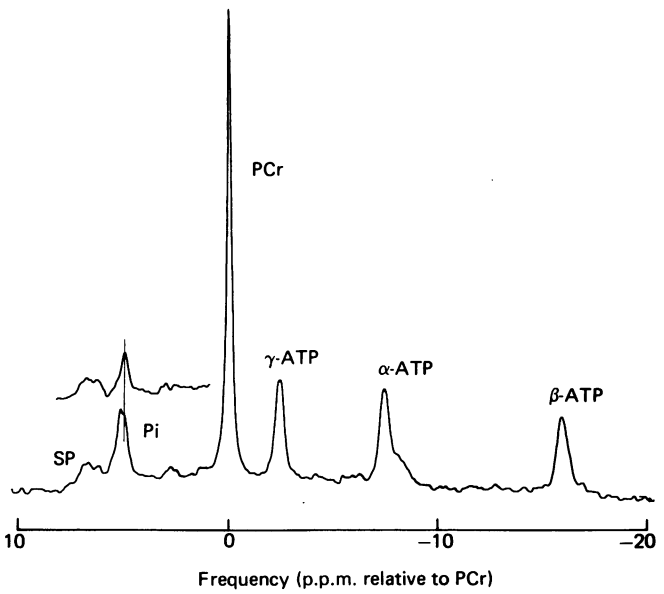

Fig. 1. ^{31}P n.m.r. spectrum from an isolated, perfused ferret heart, temperature 30 °C, stimulation rate 1·5 Hz. Ordinate, magnetic field intensity; abscissa, frequency in parts per million (p.p.m.) relative to the PCr peak. This spectrum is the average obtained from 240 pulses at 8 s intervals. The main identified peaks have been labelled (SP = sugar phosphates). The full spectrum was recorded in standard Tyrode solution (see Methods for composition), and the inset portion of a spectrum was recorded in Pi-free Tyrode solution. The vertical line drawn through the peak of the Pi peak in Pi-free Tyrode solution has a chemical shift of 5·04 p.p.m. and corresponds to a pH of 7·15.

chromatography. The mean [ATP] was 4·4 μmol (g wet wt.)$^{-1}$ (Morris *et al.* 1984). This is similar to the value found in other mammalian hearts (Dawson & Wilkie, 1984). If we assume that ATP is entirely myoplasmic, then the myoplasmic [ATP] can be calculated as follows. Taking intracellular water as 64 % of wet weight and myoplasm as 90 % of intracellular water (Jacobus, Pores, Lucas, Kallman, Weisfeldt & Flaherty, 1982) gives a myoplasmic $[ATP]_i$ of 7·5 mM. Thus, to a first approximation the values in Figs. 3 and 5 and Table 1 can be converted to myoplasmic concentrations (in mM) by multiplying by 7·5.

Measurement of pH_i using ^{31}P n.m.r.

pH_i can be calculated from the chemical shift of the Pi peak from the PCr peak. This is because the reaction:

$$H^+ + HPO_4^{2-} = H_2PO_4^-$$

has a pK_a (6·8) in the region of interest, and is in rapid equilibrium. Consequently, HPO_4^{2-} and $H_2PO_4^-$ appear as a single peak, whose chemical shift depends on $[H^+]$. PCr is used as a standard since it is relatively insensitive to changes of pH. We use the calibration of the Pi–PCr shift given by Bailey, Williams, Radda & Gadian (1981). For a discussion of possible artifacts in the measurement of pH_i with this method see Bailey *et al.* (1981).

The methods used to inhibit oxidative phosphorylation and glycolysis

Oxidative phosphorylation was prevented by two well-established techniques. In one method O_2 was replaced by N_2 by changing to a solution bubbled with 95 % N_2 + 5 % CO_2. As noted earlier, when perfused with O_2 the inflow P_{O_2} was about 600 mmHg and the effluent P_{O_2} was about 200 mmHg. When perfused with N_2, the inflow P_{O_2} was about 5 mmHg and the effluent P_{O_2} was undetectable. These figures suggest that the rate of oxidative phosphorylation was reduced by $(600-200)/(5-0)$, i.e. by a factor of about 80. In the second method HEPES-buffered NaCN was added to the standard Tyrode solution to give a final concentration of 2 mM. This was judged to be sufficient for complete inhibition as doubling this concentration did not further decrease the pressure developed (cf. Doorey & Barry, 1983). For simplicity both methods are described as producing hypoxia.

The rate of glycolysis was reduced by two independent methods. In the first method, external glucose was removed and the muscle was stimulated for a period sufficiently long to deplete the glycogen stores. In the absence of substrate, flux through the pathway is reduced. The reduction in the rate of glycolysis by this procedure was tested by subsequently inhibiting oxidative phosphorylation and showing that developed pressure fell to less than 20 % of control within 5 min (compare pressure records in Figs. 2 and 4; see also Allen & Orchard, 1983*b*). If this rate of decline of developed pressure was not achieved, the heart was reoxygenated and stimulated for a further period and then retested. By this criterion 1–2 h of stimulation in glucose-free perfusate were required to achieve an adequate reduction in the rate of glycolysis.

The reduction in the rate of glycolysis by this technique was tested independently in two experiments. Since anaerobic glycolysis produces lactate, in order to test when glycogen stores were depleted, we measured the lactate production during brief (10 min) hypoxic periods. The rate of lactate production after 5 min hypoxia with glucose present averaged 4·0 μmol (g wet wt.)$^{-1}$ min^{-1}. Immediately after glucose removal, lactate production during hypoxia fell to 1·9 μmol (g wet wt.)$^{-1}$ min^{-1}. After 1 h glucose-free stimulation it fell to 0·8 and after 2 h it fell to 0·2. In our n.m.r. experiments the average exposure to glucose-free conditions was about 2 h so that the rate of glycolysis should have been reduced more than 10-fold. This finding is consistent with biochemical measurements of glycogen stores which fall to 20 % in 60 min (Neely, Bowman & Morgan, 1969). The disadvantage of this method is that both mechanical performance and metabolic levels show some deterioration over 2 h and it is not clear to what extent this is caused by the reduction in the rate of glycolysis and to what extent it is simply time-dependent deterioration.

In the second method, glucose was removed from the perfusate and 1 mM-2-deoxyglucose was added for 30–60 min and then removed. The perfusate was maintained glucose-free after removal of deoxyglucose. N.m.r. spectra at this time show the accumulation of 10–20 mM-2-deoxyglucose-6-Pi (Fig. 4). This compound is not metabolized by phosphoglucose isomerase and acts as a competitive inhibitor of this enzyme (Wick, Drury, Nakada & Wolfe, 1956). This procedure also led to a pronounced fall in developed pressure in subsequent periods of hypoxia. The inhibition of glycolysis produced by this exposure to 2-deoxyglucose was established by measuring lactate production during brief periods of hypoxia. In two preparations the average lactate production during hypoxia before exposure to 2-deoxyglucose was 3·8 μmol (g wet wt.)$^{-1}$ min^{-1}, after 30 min exposure it was 0·81 μmol (g wet wt.)$^{-1}$ min^{-1} and after 60 min exposure it was 0·37 μmol (g wet wt.)$^{-1}$ min^{-1}. Thus, this method reduces the rate of glycolysis by 5- to 10-fold. A disadvantage of this method is that a substantial fraction of the total Pi is trapped as 2-deoxyglucose-6-Pi (20 % in Fig. 4) and this leads to a fall in the levels of other Pi containing compounds.

RESULTS

The ^{31}P n.m.r. spectrum of the ferret heart

Fig. 1 shows a spectrum from a ferret heart obtained by averaging the signal from 240 pulses at 8 s intervals starting about 30 min after the heart had been removed. This spectrum is similar to published spectra from rat hearts (Gadian *et al.* 1979). Because this spectrum was obtained from pulses at 8 s intervals, the peaks are

unaffected by saturation factors (contrast Figs. 2, 4 and 6) and thus the areas under each peak are proportional to the concentration of the metabolites. The PCr peak was the largest and was $2{\cdot}59 \pm 0{\cdot}06$ (mean $\pm$ S.E. of the mean, $n = 10$) times larger than the β-ATP peak. γ-ATP $-$ β-ATP was $-0{\cdot}03 \pm 0{\cdot}06$ times the area of the β-ATP peak,

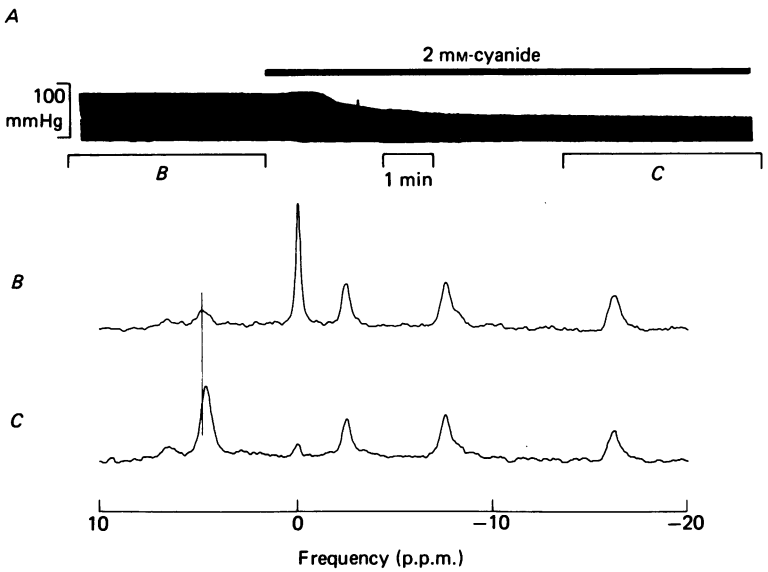

Fig. 2. The effects of hypoxia with glycolysis present. Hypoxia was induced with 2 mM-NaCN. *A*, continuous pressure record from a balloon in the left ventricle (on this slow time scale the increases in pressure associated with each beat appear fused). *B*, ^{31}P n.m.r. spectrum averaged over the 4 min immediately before the period of hypoxia. The spectrum is the average of 240 pulses at 1 s intervals. The vertical line drawn through the intracellular Pi peak has a chemical shift of 4·87 p.p.m., corresponding to a pH of 7·01. *C*, spectrum averaged over the 4 min period from the seventh to tenth minute of hypoxia.

so that ADP is not detectable. There was no identifiable AMP peak. The Pi peak in this spectrum contained contributions from both intracellular Pi and perfusate Pi so that the position of the peak (+5·21 p.p.m. relative to PCr, pH 7·33) lies between the intracellular and perfusate pH. The inset spectrum is from the same preparation after external Pi had been removed. While the rest of the spectrum was virtually identical, the Pi peak of the inset spectrum was at +5·04 p.p.m., indicating a pH_i of 7·15. A broad peak in the sugar phosphate region was detectable but variable in size between different preparations.

The effect of preventing oxidative phosphorylation when glycolysis can continue

Fig. 2*A* shows the pressure record from a ferret heart before and after oxidative phosphorylation was inhibited by 2 mM-cyanide. Glucose was present throughout so that glycolysis was able to utilize both endogenous glycogen stores and glucose. After application of cyanide there was a very small rise in developed pressure which then declined to about 50 % of control. Fig. 2*B* and *C* shows the ^{31}P n.m.r. spectra averaged over the 4 min of the control period and the seventh to tenth minute after exposure

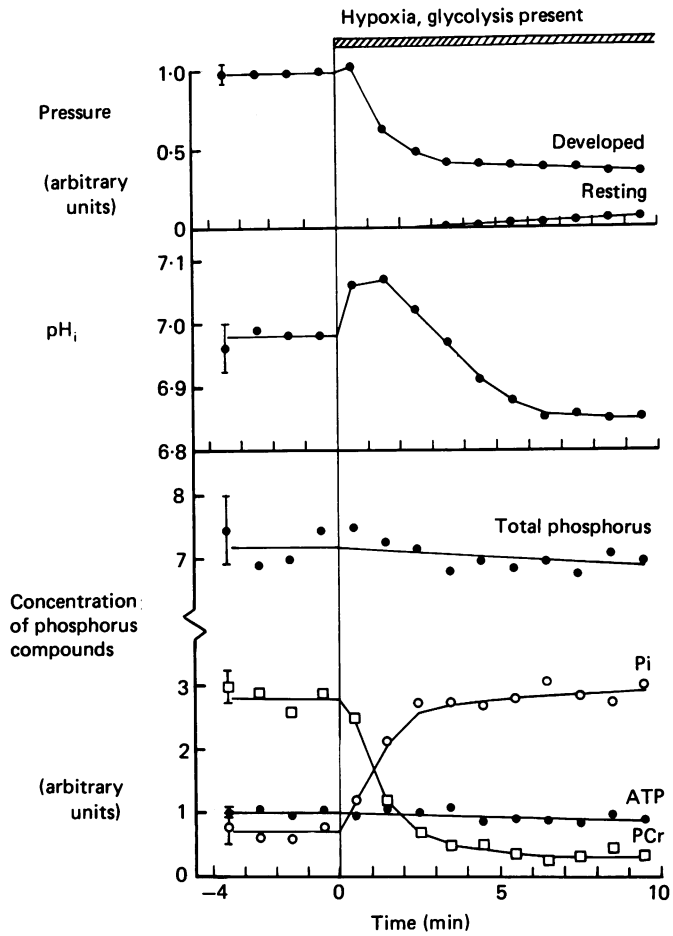

Fig. 3. Averaged mechanical and metabolic data from seven ferret hearts during hypoxia with glycolysis present. Each point represents data averaged over a 1 min period. The bars on the first point of the 4 min control period show ±1 S.E. of the mean to give an indication of the variability between preparations. The lines drawn through the points indicate the trend and have no theoretical significance. Upper panel, developed and resting pressure. Middle panel, pH_i determined from intracellular Pi chemical shift. Lower panel, [PCr], [ATP], [Pi] and total phosphorus determined from the area of peaks corrected for saturation factors. The concentrations have all been normalized to the [ATP] at the start of the experiment. Note the break in the ordinate between 3 and 7 concentration units.

to cyanide. Fig. 2*B* is similar to Fig. 1, except that saturation effects reduce the size of the peaks, particularly PCr. The line drawn through the Pi peak is at +4·87 p.p.m. indicating a pH of 7·01. The spectrum taken during hypoxia differs in that the area of the PCr peak has fallen dramatically whereas the ATP peaks show only a slight reduction. The Pi peak is substantially larger and its peak has shifted to 4·69 p.p.m., representing a pH of 6·87.

Fig. 3 shows the averaged results from seven experiments of this type with 1 min time resolution. Cyanide was used to prevent oxidative phosphorylation in six of the

TABLE 1. Summary of metabolic measurements and parameters derived from them

Concentration (arbitrary units)	Glycolysis active ($n = 7$)		Glycolysis prevented ($n = 6$)	
	Control	Hypoxia	Control	Hypoxia
PCr	2·90 ± 0·25	0·39 ± 0·05**	2·58 ± 0·31	0·27 ± 0·08**
ATP	1·00	0·88 ± 0·03**	0·72 ± 0·10†	0·44 ± 0·09**††
ADP	−0·04 ± 0·03	0·05 ± 0·04	−0·01 ± 0·04	0·09 ± 0·03
Pi	0·81 ± 0·14	3·00 ± 0·29**	1·23 ± 0·30	3·41 ± 0·61**
Total phosphorus	7·65 ± 0·39	7·24 ± 0·33*	6·94 ± 0·87	6·58 ± 0·98
pH (pH units)	6·98 ± 0·03	6·85 ± 0·03**	7·03 ± 0·02	7·00 ± 0·02††
Calculated ADP (arbitrary units)	0·0007	0·023	0·001	0·024
(μM)	5	170	7	180
Calculated ΔG_{ATP} (kJ mol^{-1})	61·5	48·6	58·9	46·9

The averaged results (mean ± S.E.) from seven experiments in which glycolysis was present are shown in columns 1 and 2. The averaged results from six experiments in which the rate of glycolysis was reduced are shown in columns 3 and 4. The control measurements were obtained from spectra of data acquired over 4 min immediately preceding the period of hypoxia. The measurements under hypoxic conditions were obtained from data acquired over the seventh to tenth minutes, inclusive, after the start of hypoxia. The concentrations of PCr, ATP, ADP, Pi and total phosphorus are in the arbitrary units defined in the methods (initial $[ATP]_i = 1$ unit). pH measurements are in pH units. Statistical comparisons between control and hypoxic parameters were made by paired t tests and significant differences are indicated by * in columns 2 and 4. Comparisons between the control conditions of the two groups of experiments were made by unpaired t tests and statistically significant differences are indicated by † in column 3. Comparisons between the hypoxic conditions in the two groups of experiments were made by unpaired t tests and the results indicated by † in column 4. * or † indicates P lies between 0·05 and 0·025; ** or †† indicates P less than 0·025. The calculated ADP figures are shown both in arbitrary units and below, in brackets, in μM and were derived as follows. The reaction catalysed by creatine kinase was assumed to be at equilibrium with an equilibrium constant of 2×10^9 M^{-1}. (This constant was determined by Lawson & Veech (1979) at 38 °C and at various free $[Mg^{2+}]$. We have adjusted their value to 30 °C, using the van't Hoff relation, and to 3 mM-Mg^{2+}.) Thus

$$[ADP] = \frac{[ATP]\,[Cr]}{2 \times 10^9\,[H^+]\,[PCr]}.$$

The total creatine ([PCr] + [Cr]) was taken as 14 μmol (g wet wt.)$^{-1}$ (Matthews *et al.* 1982; Kammermeier *et al.* 1982) which leads to 24·3 mM in the myoplasm; this was assumed to remain constant and enabled [Cr] to be calculated given [PCr]. The arbitrary units were converted to concentration units by multiplying by 7·5 (see Methods). The values of ΔG_{ATP}, the free energy change of hydrolysis of ATP, were calculated using [ATP], [ADP] and [Pi] calculated or derived as noted above. The values of ΔG^0_{ATP}, the standard free energy of hydrolysis of ATP at 30 °C and 3 mM-Mg^{2+}, were taken from Alberty (1972). These tables show the effect of pH on the standard free energy so that the values used were corrected for the changes in pH which occurred.

experiments and N_2 in one. The results of the two methods were very similar (cf. Allen & Orchard, 1983*b*). The error bars on the first point indicate ± 1 S.E. of the mean for the seven preparations. The developed pressure shows a very small rise (present in five out of seven preparations) in the first minute of hypoxia and then falls to a level of 35% by about the sixth to eighth minute. Thereafter it is reasonably stable for at least 30 min. Only a small rise in resting pressure was evident.

The pH_i averaged 6·98 in the control period and rose to a peak of 7·07 after 2 min hypoxia. pH_i subsequently declined and reached a stable level of about 6·85 after 8 min. In one experiment the pH_i remained stable at 6·85 for 30 min.

The changes in metabolite levels are shown in the lower part of Fig. 3. $[PCr]_i$ was initially about 2·9 concentration units (where initial $[ATP]_i$ is defined as

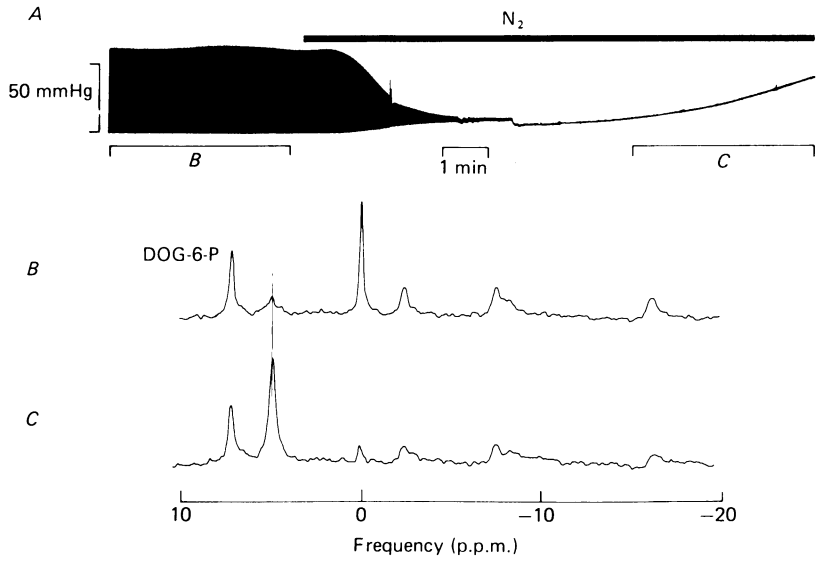

Fig. 4. The effects of hypoxia with glycolysis inhibited by 2-deoxyglucose. Hypoxia produced by replacing O_2 by N_2. The format of this Figure is the same as Fig. 2. The vertical line drawn through the peak of intracellular Pi has a chemical shift of 4·86 p.p.m., corresponding to a pH of 7·00. The 2-deoxyglucose-6-Pi peak is labelled DOG-6-P.

1 unit). This concentration fell rapidly over the first 4 min of hypoxia and reached a steady level of about 0·4 units. Over the 10 min period of hypoxia shown in Fig. 2, $[ATP]_i$ fell to about 0·9 units. Over this period Pi increased from about 0·6 units in the control to about 3 units after the 10 min period of inhibition. The sum of all the phosphorus signals fell by 0·4 units over this period. The statistical significance of these changes are listed in Table 1.

The effect of reducing the rate of glycolysis

The differences between the control period in Figs. 3 and 5 and between columns 1 and 3 in Table 1 give some indication of the maximum effects which might be attributed to the reduced rate of glycolysis. Note that developed pressure has fallen by 50 % and $[ATP]_i$ by 28 %. The other metabolites showed smaller changes which were not statistically significant (see Table 1). Both the procedures used to reduce the rate of glycolysis (see Methods) were time consuming and involved exposing the muscles to periods of hypoxia from which mechanical and metabolic recovery were incomplete. Thus, some of the above changes are attributable to aspects of these procedures rather than the reduced rate of glycolysis itself.

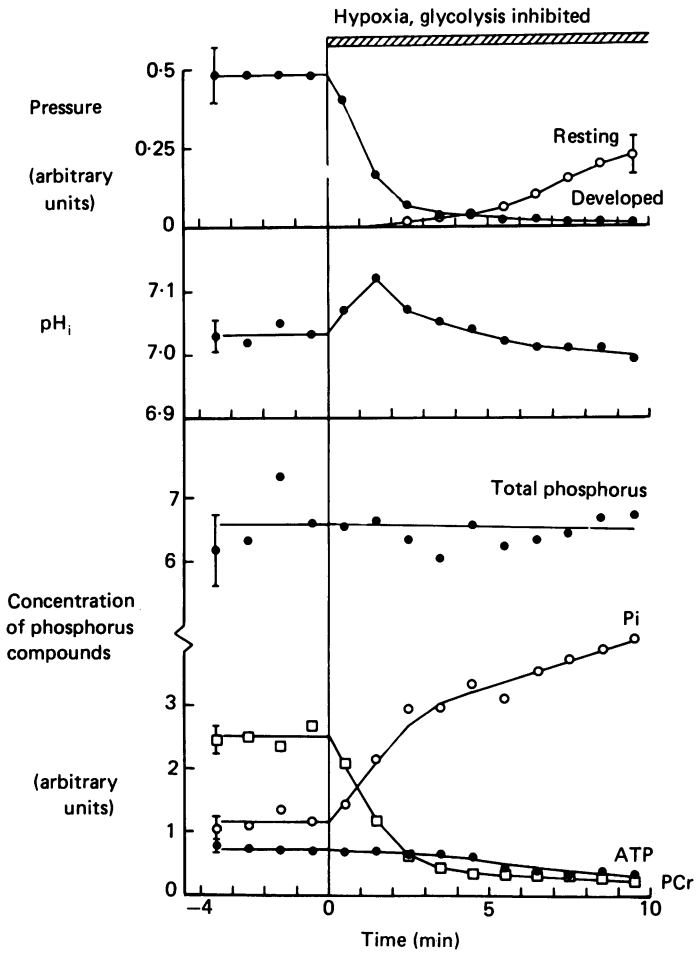

Fig. 5. Averaged mechanical and metabolic data from six ferret hearts during hypoxia with the rate of glycolysis reduced. The format of this Figure is similar to Fig. 3.

The effect of preventing both glycolysis and oxidative phosphorylation

Fig. 4 shows the effect of hypoxia, produced by N_2, in a preparation in which glycolysis had been inhibited with 2-deoxyglucose. For comparison, Fig. 6 shows an experiment in which hypoxia was induced by cyanide and the rate of glycolysis was reduced by glycogen depletion. In both Figures developed pressure falls dramatically to zero by the fourth minute of exposure and resting pressure starts to rise clearly after the sixth minute. Fig. 4 *B* and *C* shows spectra averaged over 4 min of the control period and the seventh to tenth minute under hypoxic conditions. The control spectrum is qualitatively similar to Fig. 1 apart from the large 2-deoxyglucose-6-Pi peak. The spectrum under hypoxic conditions shows a large fall in the PCr peak and in addition the three ATP peaks are all clearly reduced. The Pi peak has become very much larger but its chemical shift (4·86 p.p.m., pH 7·00) is only slightly more acid

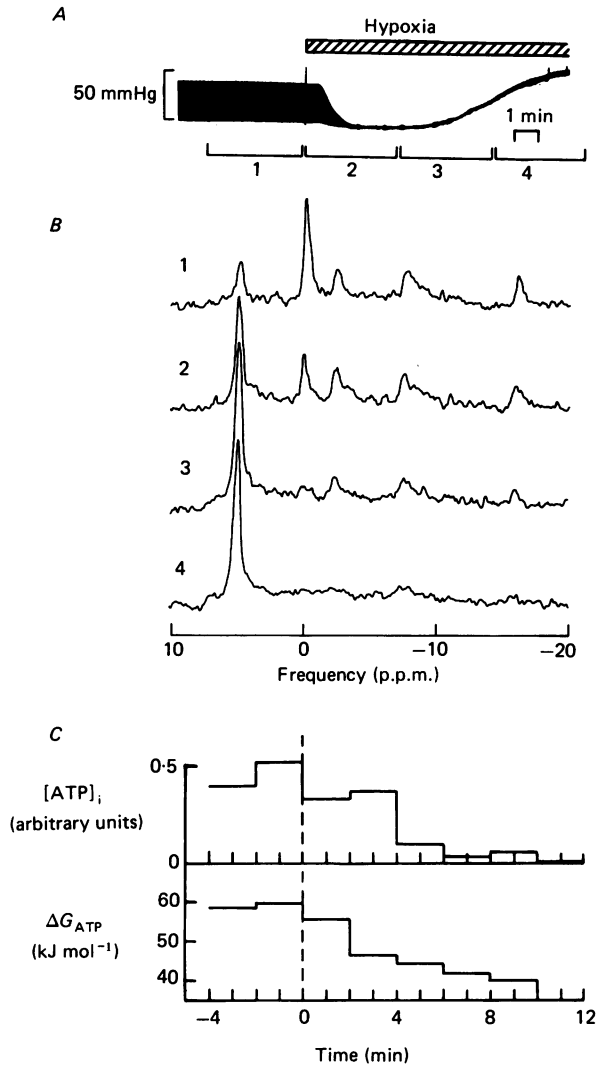

Fig. 6. The effects of hypoxia with the rate of glycolysis reduced by glycogen depletion. Hypoxia induced with cyanide. *A*, continuous pressure record. The thickening of the pressure trace during the final contracture is not due to stimulated contractions but is caused by the roller pump used to perfuse the heart. These artifacts only become apparent when the heart is stiff, as it occurs in rigor. *B*, averaged spectra from the 4 min periods indicated in *A*. *C*, upper panel, $[ATP]_i$ plotted on the same time scale as *A*. The $[ATP]_i$ (arbitrary units) have been averaged over 2 min periods. Lower panel, calculated ΔG_{ATP} using methods described in the legend to Table 1. The value in the last 2 min block was indeterminate because no ATP could be detected.

than the control peak (4·90 p.p.m., pH 7·03). The 2-deoxyglucose-6-Pi peak showed little change in its area or chemical shift.

The averaged results of six experiments are shown in Fig. 5. Hypoxia was produced by cyanide in five and N_2 in one. The rate of glycolysis was reduced by glycogen depletion in five preparations and 2-deoxyglucose in one. As described in the Methods,

these techniques reduced the rate of glycolysis on average by about 10-fold. There was no apparent difference in the results from the various methods (compare Figs. 4 and 6). Developed pressure falls rapidly under these conditions and there was no evidence of the transient rise such as shown in Fig. 2. The mean developed pressure was down to 5 % of control by the fifth minute and at about this time resting pressure was beginning to increase.

pH_i was 7·03 under control conditions, increased initially in hypoxia to a peak of 7·12 in the second minute and then slowly became more acid reaching pH 7·00 at the tenth minute. In one experiment the pH was found to become slightly more acid (by 0·01–0·02 pH units) over the next 20 min.

The $[PCr]_i$ fell rapidly under these conditions, averaging 10 % of control by the tenth minute. This level was not significantly different from the final level reached when glycolysis was present. $[ATP]_i$ fell by a factor of 2 over the 10 min period shown and continued to fall over longer periods of exposure. $[ADP]_i$ was not significantly different from zero in the control 4 min period but by the seventh to tenth minute of hypoxia it had become detectable with a value of $0{\cdot}095 \pm 0{\cdot}028$ concentration units ($P < 0{\cdot}02$). $[Pi]_i$ rose from a control value of 1·1 units to 4·0 units and was still rising at the end of the 10 min period, presumably representing the continuing fall of ATP. Total phosphorus averaged 6·94 units in the control period and 6·58 units in the seventh to tenth minutes of hypoxia, but this change was of doubtful significance on a paired t test ($P \simeq 0{\cdot}1$).

The experimental results averaged together in Fig. 5 include some in which contracture tension was small and $[ATP]_i$ did not fall to low levels, and others in which the contracture was large and $[ATP]_i$ fell to very low levels. In order to show more clearly the relation between fall in $[ATP]_i$ and contracture tension in a single experiment, Fig. 6 shows the time course of change of $[ATP]_i$ in the experiment in which the largest contracture tension was recorded. In this experiment there is a good temporal correlation between fall in $[ATP]_i$ to low levels (Fig. 6*C*, upper panel) and appearance of contracture tension. In the averaged spectrum over the last 4 min period, there are no ATP or ADP peaks detectable and all the Pi-containing compounds lie between +4 to +6 p.p.m. relative to the initial location of PCr.

DISCUSSION

The main aim of this study was to compare the metabolic changes when oxidative phosphorylation was prevented in the presence and in the absence of glycolysis. When glycolysis is present, hypoxia leads to only a moderate fall in developed pressure while when the rate of glycolysis is reduced, hypoxia leads to a greater fall of developed pressure and a contracture develops. Allen & Orchard (1983*b*) showed that in the first of these situations the amplitude of the calcium transients was unaffected while in the latter situation the amplitude of the calcium transients declined rapidly. Thus, to explain the fall of developed pressure in the first situation it is necessary to identify a metabolic change which will reduce tension production at a constant intracellular calcium concentration ($[Ca^{2+}]_i$) whereas in the second situation it is necessary to identify changes which could produce a decline in the calcium transient.

Metabolic changes during hypoxia when glycolysis can continue

It has frequently been suggested that part of the decline of developed pressure in hypoxia is a consequence of acidosis (Katz & Hecht, 1969; Tsien, 1976; Allen & Orchard, 1983*b*). It has been clearly established that acidosis reduces tension at a given $[Ca^{2+}]_i$ both in skinned (Fabiato & Fabiato, 1978) and in intact preparations (Allen & Orchard, 1983*a*). In this paper we report changes in pH_i in hypoxic conditions and consider two aspects of these changes: (i) what are the causes of the observed pH changes? and (ii) can the pH_i changes account for the observed changes in pressure?

Our pH_i measurements show an initial transient alkalosis followed by a steady acidosis. A transient intracellular alkalosis during hypoxia has not previously been reported in the heart although Hearse (1979) and Allen & Orchard (1983*b*) have suggested that PCr breakdown during the early period of hypoxia should lead to an alkalosis. The net reaction occurring in the first 2 min of hypoxia in Fig. 3 is the breakdown of PCr to Pi and Cr. According to Moisescu & Thieleczek (1978) in the reaction

$$PCr + H^+ \rightarrow Cr + Pi$$

the stoicheiometry of the H^+ involved is 0·3–0·5 under physiological conditions. On the basis of the concentration calibration suggested in the Methods, it appears from Fig. 3 that about 15 mM-PCr is broken down in the first 2 min; this should, therefore, lead to the absorption of $\sim$ 6 mM-H^+. Taking the intracellular buffering power as 70 mM (pH unit)$^{-1}$ (Ellis & Thomas, 1976), this absorption of H^+ should lead to an alkalosis of 0·09 pH units. The measured alkalosis was 0·08 pH units. The measured alkalosis would be expected to be less than that calculated above as lactate production occurs at a substantial rate after 2 min of hypoxia (Williamson, 1966).

The steady acidosis which we have detected is much less than that which has been observed in ischaemia (Bailey *et al.* 1981), presumably because lactate, which is thought to be the major cause of the acidosis, cannot leave the preparation during ischaemia. Our lactate measurements show that during hypoxia with glycolysis present about 4 μmol lactate (g wet wt.)$^{-1}$ min^{-1} is produced. The intracellular lactate concentration required to produce this flux of lactate was calculated to be $\sim$ 10 mM using the following method (unpublished experiments of J. S. Pirolo & D. A. Eisner). A heart was perfused with Tyrode solution containing a small amount of lactate for about 10 min and then lactate was removed from the perfusate and its concentration in the effluent measured at 0·5 min intervals. Lactate in the effluent was found to fall approximately exponentially with a rate constant of 0·7 min^{-1}. This rate constant was then used to calculate the intracellular lactate concentration required to produce the above efflux of lactate during hypoxia. Using the intracellular buffering power quoted above, this suggests that an intracellular acidosis of 0·15 pH units can be attributed to lactate. The observed acidosis was about 0·21, taking the peak of the alkalosis as the starting point. Thus, lactate production can account for much of the acidosis we observe.

To consider the question of whether the observed pH changes can explain the changes in tension requires measurements of tension (or better pressure) as a function of pH. These should be at constant levels of $[Ca^{2+}]_i$ since Allen & Orchard (1983*b*)

showed that the calcium transients did not change during hypoxia when glycolysis could proceed. The data of Fabiato & Fabiato (1978) show that in skinned cardiac muscle activated by a constant $[Ca^{2+}]$ to about 50 % of maximal tension, an alkalosis of 0·09 units increases tension to about 110–115 % of control, while an acidosis of 0·13 units causes tension to fall to about 70 % of control. Experiments on whole hearts in which intracellular pH was changed by varying the external $[CO_2]$ show very similar changes in developed pressure (Jacobus *et al.* 1982).

If the only factor affecting pressure was the pH_i, we would expect a transient increase in pressure to 110–115 % control in the second minute, whereas we observed (Fig. 2) a very small rise in pressure in the first minute (to 103 % control) followed by a fall to about 60 % by the second minute. In the steady state we would expect tension to fall to about 70 % of control but we observe a fall to $\sim$ 35 %.

A possible explanation for these discrepancies is that some other metabolic change also reduces tension production by the myofilaments. An attractive candidate is $[Pi]_i$ since it increases substantially during hypoxia and this increase is known to inhibit tension production in skinned cardiac muscle (Herzig & Ruegg, 1977). Our calibration procedure suggests that $[Pi]_i$ rises from about 4 to 20 mM and the data of Herzig & Ruegg suggest that this would inhibit tension production to about 65 % of the control. During the initial alkalosis $[Pi]_i$ is already substantially increased and the combined effect would be for developed tension to show little change. In the steady state both the acidosis and the increased $[Pi]_i$ will reduce the developed pressure. Thus the combined effects of pH_i changes and the increasing $[Pi]_i$ appear to adequately explain the changes in developed tension.

Metabolic changes during hypoxia when the rate of glycolysis is reduced

If both oxidative phosphorylation and glycolysis are prevented, there should be no production of ATP so its level should fall at a rate which can be calculated from the rate of consumption of ATP and knowledge of the initial $[ATP]_i$ and $[PCr]_i$ and of the equilibria which relate them. Calculations assuming that creatine kinase holds the reaction

$$PCr + ADP + H^+ \rightleftharpoons Cr + ATP$$

at equilibrium (Carlson & Wilkie, 1974) show that initially [PCr] should fall at a rate equal to Pi splitting from ATP while [ATP] should remain approximately constant. This is exactly what we observe (Fig. 5). The different rates of decline of $[ATP]_i$ and $[PCr]_i$ can, therefore, be explained by the buffering action of creatine kinase and it is not necessary to invoke cellular compartmentation, as Opie (1983) has done, to explain this observation. If ATP consumption is 1 mM s^{-1} (Gibbs, 1978), then initially $[PCr]_i$ should fall at that rate. In fact, Fig. 5 shows that the initial rate of fall of PCr is only 10 mM min^{-1} (0·15 mM s^{-1}) but this discrepancy can probably be explained as follows: (i) reduction in the rate of oxidative phosphorylation is not instantaneous, (ii) pressure development starts to fall rapidly leading to a fall in ATP consumption and (iii) the reduction in the rate of glycolysis was not complete.

If breakdown of ATP to ADP + Pi and the reaction catalysed by creatine kinase were the only reactions occurring under these conditions, $[ADP]_i$ should rise and eventually equal that of the initial $[ATP]_i$. Although, as shown in Table 1, $[ADP]_i$ does become detectable between the seventh and tenth minutes of hypoxia with

glycolysis inhibited, it is clear from Fig. 6*B* 4 that when all the ATP has been consumed it is not replaced by an equimolar amount of ADP. This suggests that ADP is further degraded by adenylate kinase to AMP and ATP and possibly also to subsequent stages in the degradative pathway (AMP → IMP → hypoxanthine). AMP or IMP, if present, would appear at about +6·3 p.p.m. in the spectrum. No such peak is clearly apparent in Fig. 6*B* 4 but the spectra from a second heart in which ATP finally became undetectable did show a substantial peak in this region. However, we have not established whether this peak is a sugar phosphate, AMP or IMP.

The cause of mechanical failure during hypoxia when the rate of glycolysis is reduced

The hypothesis that changes in pH_i contribute to the rapid decline in developed pressure is clearly untenable in hypoxia when the rate of glycolysis is reduced. Developed pressure fell to less than 5% control within 5 min but at this time pH_i was about the same as in the control period. The reason for the much-reduced acidosis is mainly that lactate production is now substantially reduced. The fall in pH_i from the peak of the alkalosis (7·12) down to 7·00 can probably be accounted for by (i) H^+ production associated with net ATP breakdown, (ii) residual lactate production because glycolysis was not completely prevented, and (iii) action of pH_i regulating mechanisms.

$[ATP]_i$ falls by only about 20% over the first 5 min of hypoxia. Our calibration suggests that this represents a fall from 6 to 5 mM. Studies of the ATP sensitivity of developed tension in skinned cardiac muscle (Fabiato & Fabiato, 1975) show that a fall of ATP of this size would have virtually no effect on tension. $[Pi]_i$ increases substantially during this period, but this rise is no greater than in hypoxia with glycolysis present so this rise can only account for tension falling to about 65% of control.

The demonstration that the calcium transients decline with a time course comparable to the decline of tension (Allen & Orchard, 1983*b*) suggests that some aspect of the metabolic change under these conditions, which differs from that when glycolysis is present, leads to failure of the calcium transient. This could be because of an effect on the action potential and membrane calcium fluxes or because calcium in some internal store was no longer present or releasable. Action potentials have been measured during hypoxia in the absence of glucose (McDonald & MacLeod, 1973) and the duration shortens very substantially over 30–60 min. However, over the 5 min in which the main fall in tension occurs, the reduction in action potential duration is only about 5%. It is possible that these changes in action potential duration would be more rapid if hypoxia was instituted in a glycogen-depleted preparation and this point requires further investigation. Recent experiments (Noma, 1983) have identified a potassium channel in the surface membrane which is activated by metabolic inhibition and probably underlies the shortening of the action potential noted above. These channels only open when $[ATP]_i$ falls below about 0·1 mM and would not, therefore, be active during the period when the rapid tension decline occurs.

It is possible that the decline in $[ATP]_i$ either prevents sarcoplasmic reticulum (s.r.) uptake or release of calcium. The K_m of the s.r. ATPase is around 20 μM (Schuurmans-Stekhoven & Bonting, 1981) so that during the period of rapid decline of tension, when we estimate myoplasmic $[ATP]_i$ to be 5–6 mM, the $[ATP]_i$ should not limit the activity of the pump.

The increase in resting pressure (hypoxic contracture) develops slowly and is still increasing after 10 min of hypoxia. In selected experiments, such as Fig. 6, it is clear that the development of resting pressure correlates well with the fall of $[ATP]_i$ to low levels. The correlation between $[ATP]_i$ falling to low levels and the appearance of resting tension is less clear in other experiments (e.g. Fig. 4) and in the averaged results shown in Fig. 5. This discrepancy is probably caused by heterogeneity of the $[ATP]_i$ in different regions of the heart and between the different preparations. For instance, if the right ventricle is working less hard than the left it may have an $[ATP]_i$ which is close to normal at a time when the $[ATP]_i$ in the left ventricle is sufficiently low to cause rigor. The net effect would be that rigor tension would be recorded from the balloon in the left ventricle although the measure $[ATP]_i$, which is spatially averaged over the whole heart, would appear greater than the level at which rigor is produced.

Free energy change of hydrolysis of ATP

Since it is generally agreed that the fall of myoplasmic $[ATP]_i$ is quite small during early hypoxic contractile failure (Gibbs, 1978; Hearse, 1979; Matthews *et al.* 1981) several groups have considered the possibility that a decline in the free energy change of hydrolysis of ATP (ΔG_{ATP}), which depends on [ADP] and [Pi] as well as [ATP], may be a causal factor (Kammermeier *et al.* 1982; Allen & Orchard, 1983*b*). The s.r. calcium pump requires a certain free energy change, derived from the hydrolysis of ATP, to pump one calcium ion from the myoplasm to the higher concentration in the s.r. Taking the myoplasmic $[Ca^{2+}]$ as 0·2 μM, s.r. $[Ca^{2+}]$ as 1 mM and assuming zero potential across the s.r. (Somlyo, Gonzalez-Serratos, Shuman, McCellan & Somlyo, 1981), the required free energy change is 21·5 kJ mol^{-1}. Since the s.r. pump transfers two calciums per ATP split (Hasselbach & Oetliker, 1983), the free energy change required of ATP hydrolysis is 43 kJ mol^{-1}. The free energy change of ATP hydrolysis is given by

$$\Delta G_{ATP} = \Delta G^0_{ATP} + RT \ln \frac{[ATP]}{[ADP]\,[Pi]},$$

where ΔG^0_{ATP} is the standard free energy change for the hydrolysis of ATP under appropriate conditions of pH, temperature and $[Mg^{2+}]$.

Dawson *et al.* (1978) estimated G_{ATP} from n.m.r. data using the following assumptions. [ATP] and [Pi] can be directly measured and their myoplasmic concentrations estimated on the basis suggested in the methods. [ADP] is generally too small to be detectable in n.m.r. spectra but its concentration can be calculated if it is assumed that the creatine kinase reaction is at equilibrium. This assumption seems likely to be true since the heart contains substantial quantities of creatine kinase and the assumption has been tested and confirmed in cardiac muscle (Matthews, Bland, Gadian & Radda, 1982). Details of the calculation and other assumptions involved are described in the legend to Table 1. Notice that the measured $[ADP]_i$ during hypoxia with glycolysis inhibited is substantially greater than that calculated using these assumptions. We do not know the reason for this discrepancy.

With these assumptions ΔG_{ATP} has been calculated and the resulting values are listed in Table 1. A large fall in ΔG_{ATP} is seen over the period of hypoxia when glycolysis is present although calcium transients over this period showed little change.

The experiments of Kammermeier *et al.* (1982) under comparable conditions (i.e. glucose present) show very similar changes in ΔG_{ATP}. However, the fall in ΔG_{ATP} is only slightly greater when glycolysis is inhibited, although under these conditions the calcium transients were abolished (Allen & Orchard, 1983*b*). Thus, these results do not appear to support the hypothesis that the decline in ΔG_{ATP} is critical to the rapid fall in tension when hypoxia occurs with glycolysis inhibited, unless there is an extremely sharp threshold at about 47–48 kJ mol^{-1}. While our results lend little support to the above hypothesis, we feel that the hypothesis should not yet be completely rejected for the following reasons. (i) Allen & Orchard (1983*a*) showed that prolonged acidosis (e.g. > 10 min) led to an increase in the calcium transients but such an increase was not apparent when the acidosis was caused by hypoxia with glycolysis present. For this reason Allen & Orchard (1983*b*) speculated that some simultaneous process was tending to reduce the calcium transients under the latter conditions. This process could be the reduced ΔG_{ATP}. Thus, we speculate that to achieve the usual loading of the s.r. requires a ΔG_{ATP} of $\sim$ 50 kJ mol^{-1} and that as ΔG_{ATP} declines the s.r. loading achieved is reduced. In hypoxia when glycolysis can continue, the ΔG_{ATP} falls below this value (ΔG_{ATP} = 48·6 kJ mol^{-1}) but the fall in calcium transients which would result is disguised by the increase in calcium transients associated with prolonged acidosis. In hypoxia when glycolysis is inhibited, the fall in ΔG_{ATP} is greater and there is no acidosis so that the calcium transients show a dramatic decline and lead to the observed rapid decline in tension. (ii) It is possible that heterogeneity between regions of the heart and between different preparations (see above) is distorting our estimates of ΔG_{ATP}. For this reason in Fig. 6*C* (lower panel) we have plotted the calculated ΔG_{ATP} over 2 min blocks for a single preparation. ΔG_{ATP} falls steadily throughout the period of hypoxia so that the ΔG_{ATP} must at some point reach the range of ΔG_{ATP} at which calcium loading of the s.r. starts to decline. However, we have not established that the fall of ΔG_{ATP} to this critical range of values coincides with the time at which the calcium transient and tension production decline.

Conclusions

When glycolysis is active, hypoxia leads to a moderate fall in tension which can be accounted for by the intracellular acidosis and increased Pi levels. When glycolysis is inhibited, hypoxia leads to a much greater fall of tension. This probably represents a failure of excitation–contraction coupling but the mechanism of this has not yet been established.

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